ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2011, p. 782–787 Vol. 55, No. 2
0066-4804/11/$12.00 doi:10.1128/AAC.01219-10

T-705 (Favipiravir) Inhibition of Arenavirus Replication in Cell Culture▽†

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Received 3 September 2010/Returned for modification 5 November 2010/Accepted 16 November 2010

A number of New World arenaviruses (Junín [JUNV], Machupo [MACV], and Guanarito [GTOV] viruses) can cause human disease ranging from mild febrile illness to a severe and often fatal hemorrhagic fever syndrome. These highly pathogenic viruses and the Old World Lassa fever virus pose a significant threat to public health and national security. The only licensed antiviral agent with activity against these viruses, ribavirin, has had mixed success in treating severe arenaviral disease and is associated with significant toxicities. A novel pyrazine derivative currently in clinical trials for the treatment of influenza virus infections, T-705 (favipiravir), has demonstrated broad-spectrum activity against a number of RNA viruses, including arenaviruses. T-705 has also been shown to be effective against Pichinde arenavirus infection in a hamster model. Here, we demonstrate the robust antiviral activity of T-705 against authentic highly pathogenic arenaviruses in cell culture. We show that T-705 disrupts an early or intermediate stage in viral replication, distinct from absorption or release, and that its antiviral activity in cell culture is reversed by the addition of purine bases and nucleosides, but not with pyrimidines. Specific inhibition of viral replication/transcription by T-705 was demonstrated using a lymphocytic choriomeningitis arenavirus replicon system. Our findings indicate that T-705 acts to inhibit arenavirus replication/transcription and may directly target the viral RNA-dependent RNA polymerase.

Several New World arenaviruses, including Junín (JUNV), Machupo (MACV), and Guanarito (GTOV) viruses, as well as the related Old World Lassa virus, are among a phylogenetically diverse group of negative-sense RNA viruses that cause severe viral hemorrhagic fevers (VHFs) in regions of the world where they are endemic (9). The National Institutes of Health has classified these viruses as category A agents because of the threat they pose to the U.S. population (20). Despite the biodefense and public health risks associated with these highly pathogenic viruses, there are no FDA-licensed arenavirus vaccines and current antiarenaviral therapy is limited to an off-label use of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), which has had only mixed success in the treatment of severe infections and is associated with significant toxicity in humans (4, 15, 27). Therefore, it is important to develop novel and effective antiviral drugs to combat arenaviral hemorrhagic fevers.

T-705 (favipiravir; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) is a pyrazine derivative with broad antiviral activity against RNA viruses, including influenza viruses (6, 16, 24, 25), flaviviruses (13, 19), bunyaviruses, and several nonpathogenic arenaviruses (10–12). Moreover, studies employing the hamster Pichinde virus (PICV) infection model of acute arenaviral disease have demonstrated that T-705 can be used effectively to treat advanced infections in animals (10). However, T-705 has not yet been tested against highly pathogenic human arenaviruses.

Evidence indicates that T-705 is ribosylated and phosphorylated to the active T-705-4-ribofuranosyl-5′-triphosphate form (T-705RTP) that inhibits influenza virus infection by interfering with viral RNA replication and transcription through inhibition of the virus RNA-dependent RNA polymerase (RdRp) (7). The broad activity of T-705 against a number of RNA viruses suggests that this inhibitor may target a conserved functional element in the viral polymerase. The ability of T-705 to specifically target the viral replication machinery may minimize the possibility of *in vivo* toxicity. In contrast, ribavirin also inhibits cellular IMP dehydrogenase (IMPDH), a key enzyme in guanosine biosynthesis, and thereby perturbs cellular nucleotide pools. In the present study, we explored the mechanism of action of T-705 in cell culture and assessed the *in vitro* activity of T-705 against three highly pathogenic arenaviruses.

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† Supplemental material for this article may be found at http://aac.asm.org/.

▽ Published ahead of print on 29 November 2010.

MATERIALS AND METHODS

Viruses. JUNV, Candid 1 strain (JUNV-C), and GTOV, strain S-26764, were provided by Robert Tesh at the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA; University of Texas Medical Branch [UTMB], Galveston, TX). JUNV, Romero strain (JUNV-R), and MACV, strain Carvallo, were kindly provided by Tom Ksiazek (Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, GA). Virus stocks of JUNV-R, MACV, and GTOV were grown in Vero (African green monkey kidney) cells.

All work with JUNV-R, MACV, and GTOV was performed under biosafety level 4 (BSL4) containment at the Robert E. Shope Laboratory at UTMB.

Tacaribe virus (TCRV), strain TRVL 11573 (ATCC, Manassas, VA), was passaged once in baby hamster kidney (BHK) cells and three times in Vero cells. The attenuated JUNV-C was passaged once in BSC-1 cells and once in Vero cells. Purified stocks were prepared for both TCRV and JUNV-C by sucrose cushion ultracentrifugation. Infected Vero cells culture lysates were clarified by low-speed centrifugation (4,500 × *g*), and the supernatants were overlaid onto a 20% (wt/vol) sucrose solution (TN buffer; 0.05 M Tris-HCl, pH 7.4, and 0.1 M NaCl) and centrifuged at 100,000 × *g* for 1 h in an SW28 rotor (Beckman Coulter, Brea, CA). The virus pellets were resuspended in phosphate-buffered saline (PBS), aliquoted, and stored at −80°C until use.

Antiviral compounds, nucleotides, and nucleosides. T-705 was provided by the Toyama Chemical Company, Ltd. (Toyama, Japan). Ribavirin was from MP Biomedical (Santa Ana, CA). Adenine, adenosine, guanine, guanosine, 2-deoxyguanosine, inosine, hypoxanthine, xanthine, cytosine, cytidine, thymine, thymidine, uracil, uridine, and uric acid were from Sigma (St. Louis, MO), and 2-deoxyadenosine, 2-deoxycytidine, and xanthosine were from ICN Nutritional Biochemicals (Cleveland, OH).

Virus yield reduction assays. For experiments evaluating drug inhibition of JUNV-R, MACV, or GTOV replication, Vero E6 (African green monkey kidney) cell cultures were infected with a multiplicity of infection (MOI) of 0.1 in duplicates in the presence of serially 2-fold diluted (1,000 to 4 uM) T-705 or ribavirin. Supernatants from infected cells were harvested at 4 days postinfection (d p.i.) for MACV, 6 d p.i. for JUNV-R, or 10 d p.i. for GTOV.

Viral titers for drug-treated JUNV-R infections were determined by plaque assay. Vero E6 cells were infected with serial 10-fold dilutions of virus for 1 h at 37°C. Cell monolayers were then overlaid with 0.5% SeaKem ME agarose (Cambrex, East Rutherford, NJ) in minimal essential medium (MEM) supplemented with 2% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S). Infected cells were cultured for 6 days, at which time a second overlay containing 1% neutral red was added. PFU were counted 18 to 24 h after addition of the second overlay, and the 90% and 50% effective concentrations (EC_{90} and EC_{50}, respectively) were calculated by regression analysis.

GTOV titers were also measured by plaque assay. Vero cell monolayers were infected with serial 10-fold dilutions of GTOV for 1 h at 37°C. After infection, cells were overlaid with 0.5% methyl cellulose in MEM supplemented with 2% FBS and 1% P/S. After a 10-day culture period, the overlay was removed, and cells were fixed with 10% buffered formalin for 20 min and stained with 1% crystal violet (Sigma). PFU were counted, and the EC_{90} and EC_{50} were calculated by regression analysis.

MACV titers were measured by a focus-forming unit (FFU) assay. Vero E6 monolayers were infected with serial 10-fold dilutions of virus for 1 h at 37°C. Following infection, cells were overlaid with 0.8% tragacanth (Sigma) in MEM supplemented with 2% FBS and 1% P/S. After infected cells were cultured for 4 days, the overlay was removed, and cells were fixed with 10% buffered formalin for 30 min and then refrigerated overnight. Fixed cells were permeabilized in 70% ethanol for 20 min and washed with PBS. Primary antibodies were diluted in PBS with 5% milk and 1% Tween 20. MACV-infected cells were incubated with primary antibody, JUNV-C antisera (kindly provided by R. Tesh, WRCEVA, UTMB), and incubated overnight at room temperature. The primary antibody was removed, and the plates were washed once with PBS. The secondary antibody, goat anti-mouse IgG labeled with horseradish peroxidase (HRP; Southern Biotech, Birmingham, AL), was diluted in PBS with 1% bovine growth serum and added to plates for 1 to 5 h at room temperature, and then the plates were washed with PBS. AEC substrate chromogen (DakoCytomation, Carpinteria, CA) was added for 15 min at room temperature. The reaction was stopped with distilled water, and fluid was removed from the wells. FFU were counted, and the EC_{90} and EC_{50} were calculated as described above.

Time-of-addition and reversal of antiviral activity assays. In time-of-addition and reversal of antiviral activity assays, Vero monolayers (70% confluent) were first inoculated with TCRV or JUNV-C. Cells and virus were incubated at 37°C for 1 h to allow virus adsorption. The inoculum was removed, monolayers were washed twice, and test medium (MEM containing 2% FBS and 50 μg/ml gentamicin) was added to the wells.

Two time-of-addition methods were employed. In method 1, monolayers were infected with TCRV or JUNV-C at an MOI of 0.2 (time zero), and T-705 was added at 1, 2, 4, 6, 8, 10, 12, or 15 h p.i. to give a final concentration of 200 μM. Cells were incubated at 37°C, and culture supernatants were collected at 24 h p.i. for virus yield determination by cell culture infectious dose assay (11). Briefly, each sample was serially diluted in 10-fold increments and plated on Vero cells in 96-well microplates. Plates were incubated for 7 days, and viral cytopathic effect (CPE) was determined for calculation of 50% endpoints (50% cell culture infectious dose [$CCID_{50}$]) as previously described (21).

In the second method, cell monolayers in triplicate wells were infected with an MOI of 0.05, and cells were treated by adding T-705 to a final concentration of 400 μM for the indicated periods (−2 to 0, 0 to 3, 3 to 6, 6 to 9, 9 to 12, 12 to 15, and 15 to 18 h p.i.). Test medium was replaced, and incubation was continued. Cells were incubated at 37°C, supernatants were collected 24 h p.i., and virus yields were determined.

Reversal of antiarenaviral T-705 activity by the addition of a molar excess of purine and pyrimidine bases and nucleosides was investigated with Vero cells infected with an MOI of 0.2 of TCRV or JUNV-C. T-705 was added to a final concentration of 200 μM; each competitive agent was added to triplicate wells to a final concentration of 400 μM. Cells were incubated at 37°C until 48 h p.i., at which time supernatants were collected and virus yields determined.

LCMV MG rescue assay. The lymphocytic choriomeningitis arenavirus (LCMV) minigenome (MG) rescue assay was used as previously described (17, 18). Briefly, BHK-21 cells were transfected with one plasmid that directs synthesis of an LCMV MG RNA expressing the firefly luciferase (fLuc) reporter gene in an antisense orientation together with two polymerase II expression plasmids encoding the L polymerase (pC-L) and nucleoprotein (pC-NP), required for MG replication and expression. The plasmid mixture was transfected at a 1:2:1 ratio of MG-fLuc–pC-L–pC-NP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). To assess potential cytotoxic effects of T-705 and ribavirin, the cells were also cotransfected with the pRL-CMV plasmid (Promega, Madison, WI) expressing the *Renilla* luciferase (RLuc) reporter gene under the control of cellular, rather than viral, transcription machinery. Four hours later, cells were reseeded into 96-well microculture dishes and incubated for 44 h with replicate serial dilutions of T-705 or ribavirin. Cells were then lysed, and fLuc and RLuc activities were detected using a dual reporter assay kit (Promega) and SpectraMax L luminometer (Molecular Devices, Sunnyvale, CA).

Reversal of T-705 and ribavirin activity in the LCMV replicon system by the addition of purine or pyrimidine bases and nucleosides was also investigated with BHK cells using the LCMV replicon system. T-705 or ribavirin was added to cells at a final concentration of 200 or 100 μM, respectively, and each base/nucleoside was added to a final concentration of 400 μM. Cells were lysed 48 h posttransfection and assayed for bioluminescence.

TABLE 1. *In vitro* inhibitory effects of T-705 and ribavirin against VHF arenaviruses[a]

Virus	Virus yield determination assay	T-705		Ribavirin	
		EC_{90} ± SD	EC_{50} ± SD	EC_{90} ± SD	EC_{50} ± SD
GTOV	Plaque	43 ± 20	15 ± 12	303 ± 228	239 ± 213
JUNV-R	Plaque	21 ± 19	12 ± 11	71 ± 81	49 ± 71
MACV	Focus-forming unit	53 ± 11	14 ± 5	122 ± 13	68 ± 21

[a] Data are the means and standard deviations (SD) from 3 or 4 separate experiments using Vero E6 cells. Values for 90 and 50 percent effective concentrations (EC_{90} and EC_{50}, respectively) are expressed in μM.

RESULTS

T-705 activity against hemorrhagic fever-causing arenaviruses. T-705 has been shown to inhibit the replication of several nonpathogenic arenaviruses but has not to date been tested for activity against the highly pathogenic viruses known to cause VHFs. Therefore, we evaluated the inhibitory activity of T-705 in JUNV-R, MACV, and GTOV infection. As shown in Table 1, T-705 was effective against GTOV, JUNV-R, and MACV at inhibitory concentrations similar to those reported for JUNV-C and other nonpathogenic arenaviruses (11). Ribavirin was also effective against the three viruses, but to a lesser degree, as reflected by higher inhibitory concentrations (Table 1) and right-shifted dose-response curves (Fig. 1) relative to T-705. Evidence of cytotoxicity by either compound was not observed at the tested concentrations.

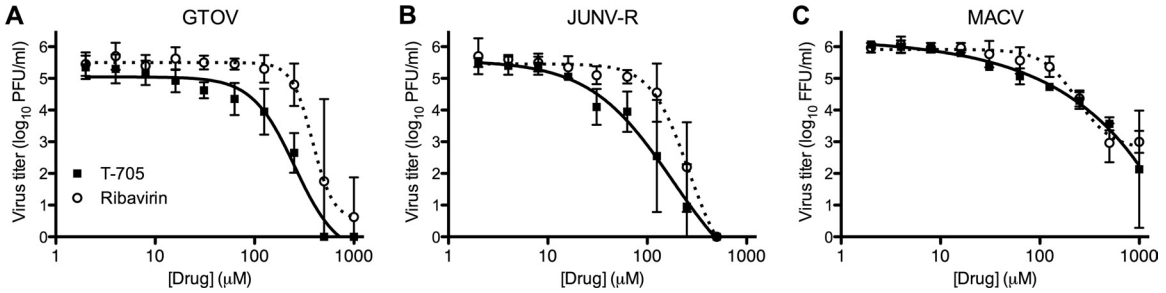

FIG. 1. T-705 inhibition of highly pathogenic arenaviruses in Vero E6 cells. The dose-response curves shown reflect the effects of T-705 (solid line) and ribavirin (hashed line) on GTOV (A), JUNV-R (B), and MACV (C) replication. The mean EC_{90}s and EC_{50}s are presented in Table 1, and the error bars represent standard deviations.

T-705 time-of-addition effect on arenavirus multiplication in cultured cells. Time-of-addition studies were conducted to assess the stage of arenaviral replication at which T-705 imparts its antiviral activity. Inhibitor was added at various times p.i., and the reduction in virus production relative to the untreated culture was assessed at 24 h p.i. In untreated cultures, infectious TCRV and JUNV-C particles could be detected in the supernatant by 14 h (not shown), suggesting an eclipse period of approximately 14 h. TCRV replication was inhibited when drug was added up to 6 h p.i. and left on throughout the 24-h incubation period (Fig. 2A, left). With JUNV-C, inhibition was seen when T-705 was withheld until as late as 8 h p.i. (Fig. 2A, right). Robust inhibition was observed generally in cultures treated within 6 to 8 h of infection. As T-705 is likely metabolized by the cell to form T-705RTP (26), these times represent minimal estimates for T-705 sensitivity. Nonetheless, the data suggest that T-705 acts at early or middle stages of the virus life cycle.

To investigate the timing of inhibition by T-705 in more detail, we conducted experiments wherein cells were exposed to the drug for short periods within the 24-h time frame of the experiment. The most robust inhibition of TCRV and JUNV-C replication was observed upon T-705 treatment during postinfection periods of 3 to 6 h, 6 to 9 h, 9 to 12 h, and 12 to 15 h (Fig. 2B). Little or no inhibition was seen when T-705 was added from −2 to 0 h, 0 to 3 h, or 15 to 18 h p.i. Taken together, these studies suggest a window for T-705 inhibition within the early and intermediate stages of virus replication, following virus entry and prior to virus assembly and budding.

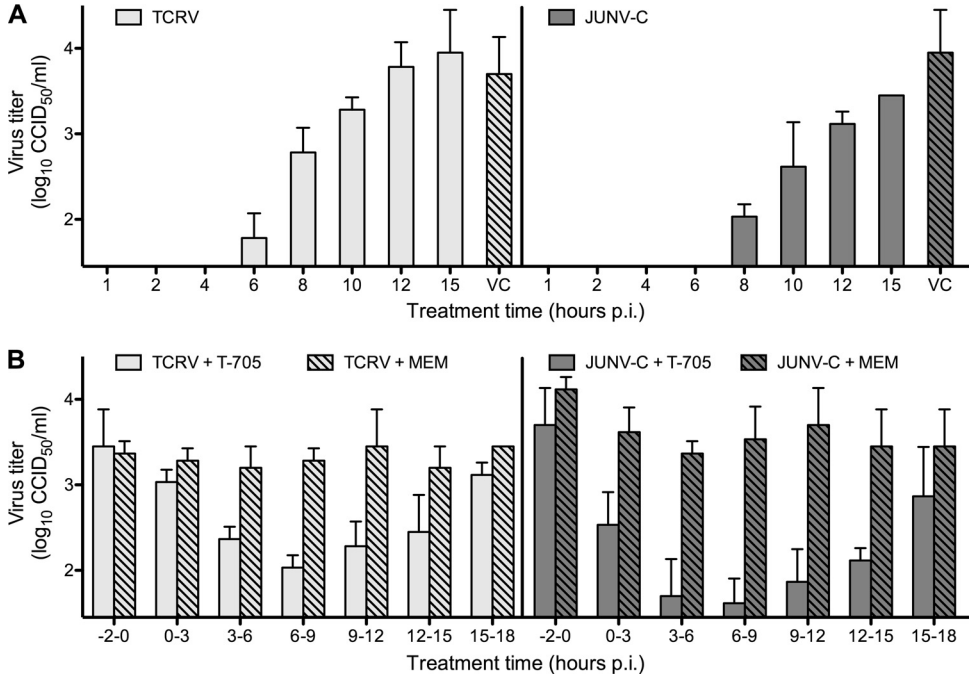

FIG. 2. Time of addition of T-705 to arenavirus-infected Vero cells. (A) For method 1, Vero cells were infected with TCRV or JUNV-C and at indicated times (*x* axis) T-705 was added to infected cells at a final concentration of 200 μM. Infected cells exposed to MEM lacking T-705 (virus control [VC]) were included for comparison. Supernatants were collected 24 h p.i., and virus yields were determined. (B) For method 2, Vero cells were infected with TCRV or JUNV-C, and at indicated time intervals (*x* axis) T-705 or MEM vehicle was added to infected cells at a final concentration of 400 μM. Supernatants were collected and virus yields determined as described above. Data shown are representative of three or four independent experiments per method for each virus, and error bars represent standard deviations for triplicate samples.

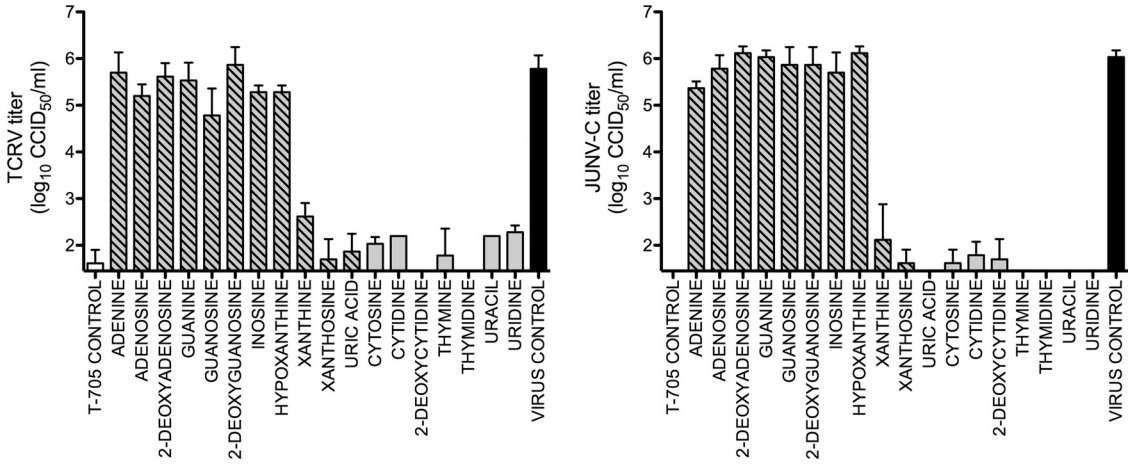

FIG. 3. Reversal of T-705 antiarenavirus activity in Vero cells infected with TCRV (left) or JUNV-C (right). Vero cells were infected with TCRV or JUNV-C, and medium containing 200 μM T-705 and 400 μM of the indicated compound (*x* axis) was added to infected cells. Supernatants were collected 48 h p.i., and virus yields determined. Patterned gray bars indicate purines, and unpatterned gray bars indicate pyrimidines or their respective nucleosides or derivatives. Black bars indicate virus controls. Results shown are representative of two independent experiments for each virus, and error bars represent standard deviations for triplicate samples.

Effects of purines at molar excess concentration on T-705-mediated anti-TCRV and -JUNV-C activity. Based on a previous study demonstrating that the antiviral action of T-705 in influenza virus-infected cells could be reversed by the addition of purines or purine nucleosides, but not by pyrimidines (7), we investigated the requirements for the reversal of T-705 activity in arenavirus infection. As seen in Fig. 3, TCRV and JUNV-C production could be rescued from T-705 action by the addition of a molar excess of purines, including adenine, adenosine, 2-deoxyadenosine, guanine, guanosine, 2-deoxyguanosine, inosine, and hypoxanthine. In contrast, compounds generated in purine catabolism (xanthine and uric acid) and xanthosine did not reverse the action of T-705. Likewise, the pyrimidine nucleobases (cytosine, thymine, and uracil) and nucleosides (cytidine, 2-deoxycytidine, thymidine, and uridine) had little or no impact on T-705 antiarenavirus activity.

Effect of T-705 on the activity of an LCMV MG. Previous studies have shown that arenavirus replication can be modeled using a recombinant plasmid replicon system comprising the viral RdRp (L), the nucleoprotein (N), and an RNA MG (5, 17, 18, 23). To specifically investigate the effects of T-705 on viral replication and transcription, we made use of the LCMV replicon system. In this assay, RdRp-dependent replication of the antigenomic viral RNA is evidenced by expression of a firefly luciferase (fLuc) reporter gene in the MG RNA. Inhibition of fLuc expression in cells transfected with the three-plasmid replicon would be consistent with a disruption of RdRp function. As shown in Fig. 4A, transcription from the LCMV replicon system was inhibited by T-705 (EC_{50} of 29 μM). Cell-driven expression of a cotransfected *Renilla* luciferase (RLuc) plasmid, which provides a measure of the effects on cellular transcription, was minimally affected at the highest concentrations of T-705 tested (Fig. 4B). This result demonstrates the specificity and apparent absence of general cytotoxicity by T-705. Ribavirin was also shown to inhibit fLuc expres-

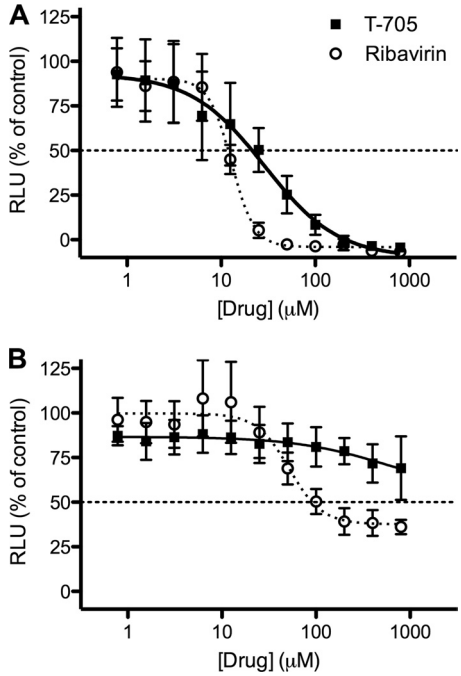

FIG. 4. T-705 inhibition of LCMV replicon system. BHK cells transfected with the LCMV replicon system (fLuc) and control (RLuc) plasmids were exposed to serial dilutions of T-705 or ribavirin. After 44 h in culture, the cells were lysed, and fLuc and RLuc luminescence was detected by dual luciferase reporter assay. fLuc expression reflects L- and N-dependent replication and transcription from the LCMV MG (A), and RLuc reporter activity is representative of cellular transcription and is a measure of cell viability (B). Results are compiled from three independent experiments and are presented as percentages of untreated controls. Error bars represent standard deviations. RLU, relative light units.

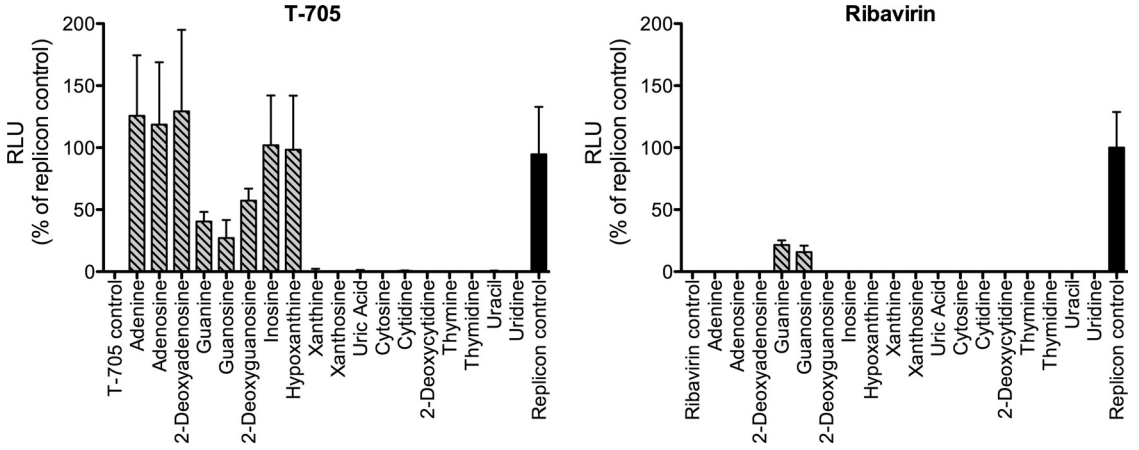

FIG. 5. Reversal of T-705 LCMV replicon inhibition. Following transfection as described in the legend to Fig. 4, 400 μM of the indicated compounds (*x* axis) was added to the cells with 200 μM T-705 (A) or 100 μM ribavirin (B). Cells were lysed after a 44-h culture period. Patterned gray bars indicate purines and their respective nucleosides or derivatives, and unpatterned gray bars indicate pyrimidines. Data are presented as percentages of untreated replicon controls (black bars). Results shown are representative of two independent experiments, and error bars represent standard deviations calculated from a minimum of 4 replicates. RLU, relative light units.

sion by the LCMV replicon (EC_{50} of 13 μM), but considerable cytotoxicity (50% cytotoxic concentration [CC_{50}] of ~100 μM) was also observed (Fig. 4B). This cytotoxic effect likely contributes to the unusually steep dose-response curve observed for ribavirin (Fig. 4A) and artifactually reduces its EC_{50}.

Consistent with our antiviral studies, the inhibitory action of T-705 was also reversed by purines and purine nucleosides when assessed using the LCMV replicon system. Significant rescue from inhibition was provided by adenine, adenosine, 2-deoxyadenosine, 2-deoxyguanosine, inosine, and hypoxanthine (Fig. 5). Guanine and guanosine also reversed the effect of T-705, albeit to a lesser extent. The pyrimidines, as well as xanthine, xanthosine, and uric acid, were again inactive in this assay. Inhibition by ribavirin was also partly reversed by guanine and guanosine. In contrast to T-705, however, the addition of adenine or adenosine did not prevent inhibition by ribavirin. This is consistent with the known inhibitory effect of ribavirin on the cellular IMPDH, which is not involved in adenosine biosynthesis. This observation suggests that the target for inhibition by T-705 is distinct from that of ribavirin, which appears to act predominantly to inhibit IMPDH and the synthesis of GMP.

DISCUSSION

T-705 has demonstrated remarkably broad *in vitro* activity against a range of RNA viruses (see Table S1 in the supplemental material). For many of these viruses, treatment options are severely limited, and in the case of influenza virus, oseltamivir resistance remains a concern (1). In particular, therapeutic options for treating severe arenaviral hemorrhagic fever cases are restricted to the use of ribavirin (2) or, in the case of Argentine hemorrhagic fever, to transfusion of immune plasma. Safer and more effective countermeasures are clearly needed (4, 15). T-705 is currently being evaluated in clinical trials in Japan and the United States for use in the treatment of influenza virus infections (8). FDA approval for the safe use of T-705 for influenza virus infection would facilitate its development for other RNA virus treatment indications. Here, we have demonstrated for the first time that T-705 is active against the highly pathogenic human arenaviruses JUNV-R, MACV, and GTOV and provided evidence that suggests that T-705 may act as a purine nucleoside analog specifically targeting arenaviral RdRp.

A recent study exploring the mechanism of action of T-705 against influenza virus infection suggests that the viral polymerase is the principal target of the active T-705 metabolite T-705RTP (7). We hypothesize that T-705 is also able to inhibit arenavirus multiplication by targeting the virus polymerase complex. It has been shown that influenza virus replication is inhibited by T-705 at an early or middle stage of infection and that purines but not pyrimidines are able to competitively reverse anti-influenza virus activity (7). In the present study, we observed analogous results in arenavirus infection.

In our studies of the reversal of T-705 inhibition, nearly all purine-based compounds showed a significant effect on T-705 activity. The notable exceptions were uric acid, xanthine, and xanthosine. Uric acid is the end product of purine degradation and would thus not be expected to affect inhibition by T-705. The biological consequences of xanthine and xanthosine metabolism are poorly defined. Indeed, all biosynthetic and catabolic purine pathways in the cell are highly interconnected and tightly regulated, making it difficult to ascribe a specific mechanism for the reversal of T-705 inhibition. However, in *in vitro* assays of influenza virus RdRp activity, GTP has been shown to be competitive with T-705RTP (7). Further biochemical studies are needed to test the leading hypothesis that T-705 acts as a nucleoside analog to inhibit the arenaviral RdRp. Additional information from the analysis of T-705 resistance will also be helpful in identifying the precise viral target.

Inhibition of the LCMV MG system indicates that T-705 interferes with virus transcription and/or replication. The molecular mechanism for inhibition, however, is not known and may include effects on L, NP, or MG. Cellular transcription, as measured by the RLuc reporter, was unaffected. In contrast,

ribavirin demonstrated significant inhibition of cellular processes at concentrations only slightly greater than those that inhibit the LCMV replicon. This is consistent with its known inhibition of IMPDH (7, 28) and its recognized *in vivo* toxicity (3, 22). The ability of hypoxanthine to reverse inhibition by T-705, but not by ribavirin, provides additional evidence that T-705 does not inhibit cellular IMPDH (29). The specific inhibitory activity of T-705 against South American VHF viruses and its apparent lack of cellular toxicity bode well for further development of T-705 in the treatment of severe arenaviral hemorrhagic fevers.

ACKNOWLEDGMENTS

J.H.N. is grateful to Ana Sanchez (The Scripps Research Institute) and Sudhakar Agnihothram (The University of Montana) for facilitating implementation of the minigenome rescue assay.

This work was funded by subawards to B.B.G. and J.H.N. as part of National Institutes of Health (NIH) grant U54 AI-065357 (Rocky Mountain RCE; J. Belisle, principal investigator [PI]) and supported in part by NIH contract N01 AI-30048 (D.F.S., PI). J.-C.D.L.T. was supported by NIH grant R01 AI-075298.

Y.F. is an employee of the Toyama Chemical Co., Ltd., the manufacturer of T-705. All other authors declare no conflict of interest.

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